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(54) Title: INFANT FORMULA AND INFANT FORMULA ADDITIVES

(57) Abstract

Disclosed are infant formulas, infant formula additives, and pharmaceutical formulations that include active lactoferrin combined with antibodies which specifically bind IgA protease-like proteins or IgA protease precursor-like proteins. The formulas protect persons who drink the formulas or formulations from the pathogenic effects of bacteria and other infectious agents, e.g., viruses, which infect the upper respiratory tract.

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INFANT FORMULA AND INFANT FORMULA ADDITIVES

Background of the Invention

This invention relates to infant formulas and infant formula additives that help protect against infection.

Adults have high concentrations of IgA in their secretions, and this immunoglobulin isotype defends against infection at mucosal surfaces. In contrast, the normal newborn child has no mucosal IgA of its own. To make up for this deficiency, human milk is rich in IgA (averaging about 120 mgm/dl, but varying widely through the months of lactation), and provides passive immunity by bathing the infant mucous membranes, e.g., the oral cavity, pharynx, bronchi, and intestine with antibodies. Human milk IgA antibodies have specificity reflecting the antigen experience of the mother, and those antigens to which the child is also likely to be exposed. During the first six months of life, however, IgA slowly appears in the child, carrying antibody specificity for bacteria, viruses, food proteins and other mucosal antigens. Therefore, the maternal/infant collaboration consists of initial "passive" antibodies from the mother's milk during the period in which the child's secretory immune system matures, and then the child's own "active" antibodies.

Bacterial colonization involves the attachment and proliferation of bacteria to mucosal surfaces. Under certain circumstances these microorganisms cause disease. Certain bacteria that colonize and infect infants continuously secrete IgA proteases that can cleave both maternal and infant IgA. For example, all strains of *Haemophilus influenzae* (HI), *Neisseria meningitidis*, and *Streptococcus pneumoniae* that have been studied produce these IgA proteases and cause serious infections in

children. Conditions caused by these bacteria include otitis media, pneumonia, bronchitis, sinusitis, septicemia and meningitis. These bacterial infections are a major problem in all countries and even in major U.S. cities. In the United States, for example, 67% of all children develop otitis media by the age of three years (Mandell et al., Principles and Practice of Infectious Disease (3d ed.), p. 506, Churchill Livingstone, N.Y. 1990). HI is the predominant pathogen that causes otitis media.

IgA proteases are encoded as a single large polypeptide chain by *iga* genes in the bacterial chromosome. The proteases are secreted from bacterial cells by an autocatalytic processing mechanism. Bacterial IgA proteases cleave and inactivate human IgA on mucosal surfaces and, in this way, allow bacterial pathogens to evade the mucosal immune mechanisms. IgA cleavage impairs function by dissociating the Fab and Fc regions of IgA. In addition, free Fab fragments may also bind key microbial antigens, blocking access by other protective antibodies.

Certain infant formulas have been designed to protect the infants that drink the formula against bacterial and rotavirus infections. Modification of infant formula typically involves immunizing a pregnant cow with certain bacteria or their toxins, and then extracting the desired antibody from the colostrum (the first milk after calving) for addition to formula products. Such antibodies are known to protect children against infection by several intestinal pathogens including enterotoxigenic *E. coli*, *Vibrio cholerae*, and rotaviruses.

Summary of the Invention

Applicants have discovered that, in the presence of lactoferrin, IgA protease precursor undergoes an alternative form of processing, which renders the entire IgA protease precursor susceptible to neutralizing antibodies which specifically bind the precursor.

Accordingly, the invention features, in one aspect, an infant formula which includes pasteurized milk (e.g., colostrum), active lactoferrin (e.g., non-pasteurized lactoferrin), and an antibody which specifically binds (i.e., inhibits the proteolytic activity of) either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor.

In a second aspect, the invention features a method for preparing a pasteurized infant formula. The method involves immunizing a non-human mammal with all or a portion of an IgA protease or an IgA protease precursor; collecting from the mammal milk (e.g., colostrum) including antibodies which specifically bind (i.e., inhibit the proteolytic activity of) an IgA protease or an IgA protease precursor, or both; pasteurizing the milk; and preparing the infant formula using the pasteurized milk and active (e.g., non-pasteurized) lactoferrin.

The invention also features a method for producing an infant formula additive, involving immunizing a pregnant non-human mammal with all or a portion of an IgA protease or an IgA protease precursor; collecting from the mammal milk (e.g., colostrum) including antibodies which specifically bind (i.e., inhibit the proteolytic activity of) an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor; pasteurizing the milk; and mixing pasteurized milk with

active (e.g., non-pasteurized) lactoferrin to produce the infant formula additive.

The invention further features a method for inhibiting processing of an IgA protease precursor on a cell. This method involves contacting the cell with a formulation comprising a pasteurized excipient, and active (e.g., pasteurized) lactoferrin, thereby producing an extracted IgA protease precursor; and contacting the extracted IgA protease precursor protein with an antibody which specifically binds (i.e., inhibits the proteolytic activity of) the IgA protease precursor.

In yet another aspect, the invention features a method for inhibiting proteolysis of an IgA1 antibody by an IgA protease. This method involves contacting a cell which includes a precursor of the IgA protease with a formulation that includes a pasteurized excipient, and active (non-pasteurized) lactoferrin, thereby producing an extracted IgA protease precursor. The method also involves contacting the extracted IgA protease precursor with an antibody which specifically binds (i.e., inhibits) the proteolytic activity of the IgA protease.

Also within the invention is a pharmaceutical formulation which includes a pasteurized excipient; active (e.g., pasteurized) lactoferrin; and an antibody that binds (i.e., inhibits) the proteolytic activity of an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor.

Similarly, the invention includes a pharmaceutical formulation comprising active (e.g., pasteurized) lactoferrin and an antibody that specifically binds (i.e., inhibits) an IgA protease-like protein, an IgA protease precursor-like protein, or both an IgA protease-like protein and an IgA protease precursor-like protein.

Finally, the invention features a monoclonal or polyclonal antibody that specifically binds an IgA

protease precursor and that does not specifically bind an IgA protease. For example, an antibody that specifically binds the helper section of an IgA protease precursor is included within the invention.

In preferred embodiments, the infant formula includes non-human milk or colostrum, such as cow's colostrum. As used herein, the term "milk" encompasses colostrum and the subsequent lacteal secretions. Preferably, the formula includes colostrum (i.e., the antibody-rich first secretion at the termination of pregnancy). In a preferred embodiment, the immunization is performed during the last month of gestation, and the antibodies are collected by collecting colostrum. Preferably, the animal (e.g., cow) is immunized during each of a first and a second pregnancy, and the colostrum or milk is collected after the termination of the second pregnancy since colostrum or milk collected at that point has higher levels of the desired antibodies. Preferably, the antibody which specifically binds an IgA protease precursor-like protein binds a vacuolating cytotoxin (e.g., from *Helicobacter pylori*), a Tsh protein (e.g., chicken *E. coli* Tsh protein), or a hemagglutinating adherence protein (HAP protein).

The lactoferrin which is employed in any of the various aspects of the invention can be derived from any mammal; preferably, the lactoferrin is human or bovine in origin. Lactoferrin from other ruminants (e.g., sheep and goats) is also preferred. Such lactoferrin can be isolated from a secretion (e.g., milk) of the mammal, or the lactoferrin can be produced with recombinant DNA techniques using standard techniques. Recombinant lactoferrin used in the invention can be produced *in vitro* or *in vivo*. For example, lactoferrin produced in cultured mammalian tissue is suitable in the invention. Lactoferrin produced in a prokaryotic cell can also be

used in the invention since there is no apparent requirement for the carbohydrates found in naturally occurring lactoferrin. In addition, conventional gene expression methods can be used to overexpress lactoferrin (e.g., in yeast, baculovirus, or in a mammal (e.g., a transgenic cow)), and lactoferrin obtained from such a mammal can be used in the invention.

If desired, colostrum or milk used in the invention can be processed before preparing the infant formulas of the invention. For example, fat and casein may be removed. As is discussed herein, processing steps which include heating or acidification should not be performed after addition of lactoferrin to a formula. If desired, processing can include the step of reducing the volume of the colostrum by filtering through a 100,000 MW filter. In addition, antibodies (e.g., IgG) can be purified from milk, combined with lactoferrin, and used as a formula additive.

As used herein, the term "infant formula" includes colostral or other milk preparations that include the inhibitors or antibodies of the invention and, if desired, purified inhibitors that are fed to an infant orally in combination with any other suitable beverage.

The term "antibody" includes not only complete antibodies but immunologically-active fragments thereof, which include any antibody fragments that are effective to inhibit the proteolytic activity of IgA proteases, IgA protease-like proteins, IgA protease precursors, or IgA protease precursor-like proteins. Antibodies raised against the entire protease precursor-like protein, the helper section of the precursor-like protein, or the mature protease-like protein all are included. Also included are monoclonal antibodies that specifically bind any portion of the IgA protease-like protein or

precursor-like protein, and which inhibit the proteolytic activity of the protein.

As used herein, the term "protease" includes not only the complete protease but immunologically-active fragments thereof, which include any peptide fragments that give rise to antibodies, e.g., against the complete IgA protease, when used as an immunogen.

By "active" lactoferrin is meant lactoferrin which retains the ability to extract an IgA protease precursor or protease precursor-like protein from the membrane of a cell. Generally, lactoferrin is active if it is purified or produced without substantial heating (substantial heating is considered incubation at 56°C for 30 minutes or 90°C for 5 minutes) or acidification (incubation at pH 4.0 or below). Generally, conventional pasteurization techniques (e.g., heating milk for 30 minutes at 68°C) inactivate lactoferrin. Generally, lactoferrin is inactive in the presence of 15 mM EGTA or EDTA. As used herein, the term active lactoferrin includes derivatives of, and those portions of lactoferrin that retain the above-described extraction activity. The lactoferricin domain of lactoferrin is not in and of itself considered active lactoferrin because it is insufficient to extract the protease precursor. Active lactoferrin can be produced with recombinant DNA techniques (e.g., purification from a eukaryotic cell engineered to express lactoferrin *in vitro*). Alternatively, the exogenous lactoferrin can be purified from a natural source of lactoferrin. For example, purified active bovine lactoferrin can be admixed with pasteurized bovine colostrum or milk containing appropriate antibodies in preparing an infant formula of the invention. Alternatively, the active lactoferrin may be derived from a species (e.g., a cow) which is different from the

species (e.g., a goat) from which milk or colostrum of the formula is derived.

The invention offers several advantages. By using lactoferrin to extract the IgA protease precursor-like protein from the cell membrane, and by using antibodies directed against the protease precursor-like protein or mature protease-like protein, the invention provides a means for inhibiting every IgA protease or protease precursor-like protein in a sample. By so doing, every IgA1 antibody of every specificity in the person (e.g., infant) will be available to contribute to a protective immune response. This type of protection against the bacterial IgA protease does not require killing the bacterium. In addition, immune complexes formed by antibodies that bind the IgA protease or its precursor can be delivered to M cells for generation of an immune response more readily than is the IgA protease or protease precursor in the absence of such antibodies. Applicants have also found that IgA protease precursors from gram negative cells and proteases from gram positive cells are extracted by lactoferrin and thus are both susceptible to neutralizing antibodies. This effect of lactoferrin is unaffected by iron-saturation of the lactoferrin, thus expanding the applicability of the method.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Detailed Description

The drawings are first briefly described.

Drawings

Figs. 1A and 1B are photographs of gels showing that the IgA protease precursor is extracted when the cells are grown in whey.

Fig. 2 is a photograph of a gel showing that the IgA protease precursor is extracted when the cells are grown in substantially pure human lactoferrin.

Fig. 3 is a photograph of a gel showing that the IgA protease precursor is extracted when the cells are grown in pure, recombinant lactoferrin.

Figs. 4A to 4C are photographs of gels showing that processing of the extracted IgA protease precursor is inhibited in the presence of secretory IgA1 (sIgA1).

Fig. 5 is a chart showing the titers obtained after immunization of 18 cows with IgA proteases.

Fig. 6 is a chart showing the titers obtained after a second immunization and calving.

Fig. 7 is a chart providing a second example of titers obtained after immunization of cows with IgA protease.

Figs. 8A and 8B are graphs showing the difference in inhibiting titre against various IgA proteases having the same or different serogroups as the immunizing enzyme.

Fig. 9 is a chart providing the protease inhibition titers of cows after a second set of immunizations.

Fig. 10A is a reproduction of an autoradiogram of an electrophoresis gel showing the activity of IgA protease when added to infant formulas.

Fig. 10B is a reproduction of an autoradiogram of an electrophoresis gel showing the inactivity of IgA protease when added to infant formulas supplemented with the bovine antibodies of the invention.

PROCESSING OF IgA PROTEASE PRECURSOR AND PRECURSOR-LIKE PROTEINS

Normal Processing of an IgA Protease Precursor-like Protein: In conventional growth media, IgA protease precursor-like proteins are proteolytically processed into the mature enzymes in reactions that may be autocatalytic in nature. For example, the IgA protease precursor of HI exists as a 184 kD transmembrane protein that undergoes autocatalytic cleavage to produce a 109 kD secreted enzyme. The 70-80 kD helper section (i.e., beta domain) remains docked in the outer membrane of the cell. On an SDS polyacrylamide gel, the observed sizes of these proteins is larger than the predicted sizes. The IgA protease precursor migrates as a species of 210 kD; the secreted enzyme migrates as a species of 116 kD; and the helper section migrates as a species of 120 kD.

Alternative Processing of an IgA Protease Precursor-like Protein: Applicants have discovered that, when bacteria expressing a cell-surface IgA protease precursor-like protein are contacted with lactoferrin, the IgA protease precursor-like protein undergoes an alternative form of processing. For example, when an HI cell containing an IgA protease precursor is treated with lactoferrin, the entire protease precursor is extracted from the cell. Extraction of the entire protease precursor occurs at only low levels (10-20%) in normal growth media or buffer lacking lactoferrin. In the absence of inhibitory antibodies, the extracted protease

precursor can be further processed to release the protease. This processing occurs at a site (the C site) distinct from the processing site of the membrane-bound protease precursor (the A site). In the presence of inhibitory antibodies, processing of the extracted protease precursor at the C site is blocked. Experiments demonstrating this phenomenon are described below.

Extraction of IgA Protease Precursor in Whey:

This experiment demonstrates that the IgA protease precursor of HI undergoes alternative processing when a normal milk whey (i.e., de-fatted milk) is used as the culture medium. This experiment employed a strain of *Haemophilus influenza* (HI) that has a mutation in the *iga* gene encoding the IgA protease precursor. The mutant IgA protease precursor is unable to cleave the mature protease off of the protease precursor.

In this experiment, the mutant HI cells were grown in a BHI medium until they reached mid-log phase (approximately 5 hours). Several 0.4 ml aliquots of cells were taken from the culture, and the cells were washed once with saline. The 0.4 ml aliquots of cells then were grown in 0.2 ml whey for various lengths of time (2, 5, 10, 20, 40, or 80 minutes). As a control, an aliquot of cells was grown in saline for 80 minutes. At the ends of the incubation periods, the cells were harvested by centrifugation, and 25% of the cell pellet and 100 μl of the milk supernatant from each culture were analyzed by western blot analysis using antibodies raised against gel-purified the IgA protease.

Growth of HI cells in milk results in extraction of the IgA protease precursor from the cell surface into the culture supernatant. As is illustrated in Figs. 1A and 1B, over time, the IgA protease precursor becomes undetectable in the cell pellet, and it appears in the culture supernatant (Figs. 1A and 1B; compare lanes 2-7).

In addition, the ability of whey to extract the IgA protease precursor from the cell is proportional to the concentration of whey in the sample. Dilution of the whey led to a diminished ability to extract the IgA protease precursor from the cell. In contrast to the behavior of the IgA protease in milk, the protease was not extracted when cells were grown for 80 minutes in saline (Figs. 1A and 1B, lane 8). These data thus indicate that a component of milk promotes extraction of the entire IgA protease precursor from the cell.

In this experiment, the predominant protein species detected with anti-IgA protease antibodies migrated through an SDS-polyacrylamide gel as though it were approximately 240 kD in size. A less prevalent species also was present, and it migrated as though it were 190 kD in size, the predicted molecular weight of the IgA protease precursor. Several lines of evidence indicate that the species migrating as 240 kD and 190 kD both are related to the protease precursor.

To determine whether the apparent shift in the molecular weight was due to glycosylation, the protein was treated with glycosidases. The negative results in these experiments suggest that glycosylation is not likely to be the cause of the apparent shift in the mobility of the protein. We also examined the possibility that the apparent molecular weight is caused by an adduct from the milk in which the HI cells were grown. To test this possibility, antibodies were raised against the extracted 240 kD protein. These antibodies then were used to probe a sample of milk. However, nothing in milk crossreacted with the antibodies, suggesting that milk does not contain an adduct responsible for the apparent molecular weight of the extracted IgA protease precursor. In addition, no adduct

was detectable in an extraction reaction that included ^{125}I-labeled milk proteins.

In contrast, these same antibodies specifically bound the IgA protease, the IgA protease precursor, and a recombinant helper section of the IgA protease precursor. In addition, these antibodies crossreact with the 210 kD unprocessed IgA protease precursor of an HI strain which has a mutation in the *iga* gene. These results indicate that the protein which migrates as a 240 kD species is the extracted IgA protease precursor. The data also indicate that the helper section is responsible for the apparent shift in the molecular weight of the IgA protease precursor.

When cells are grown in a conventional culture medium in the absence of lactoferrin, the enzyme portion of the IgA protease is no longer attached to the membrane; however, the helper section remains in the membrane. Treatment of cells having a membrane-bound helper section with whey leads to extraction of the helper section of the IgA protease precursor. Treatment of cells having an intact membrane-bound protease precursor with whey leads to extraction of the unprocessed protease precursor. Like the full-length extracted IgA protease precursor, the extracted helper section exhibits a decrease in mobility by SDS-PAGE. Taken together, these data lead to the conclusion that growth of HI in whey leads to extraction of the entire IgA protease precursor or the helper section of the protease precursor from the outer membrane of the cell. Although most of the extracted protein migrates with a decreased mobility by SDS-PAGE, the antibody crossreactivity of the extracted species indicates that the extracted protein having an apparent molecular weight of 240 kD is the extracted IgA protease precursor. In addition, N-terminal sequencing of the extracted proteins

confirms that the N-termini of both the 190 kD and 240 kD extracted species match the N-terminus of the IgA protease precursor. These and other data also suggest that the 190 kD protein is a degradation product of the extracted 240 kD protease precursor.

Identification of the Extracting Agent: Several lines of reasoning suggested that a milk lipid was the factor responsible for extraction of the IgA protease precursor from the cell membrane. Milk lipids have antimicrobial properties, and they have been shown to release proteins from cell membranes. In addition, milk lipids are able to modify proteins and increase their apparent molecular weight. To determine whether the extracting agent was a lipid, we treated whey with acetone to precipitate the proteins. Unexpectedly, the precipitate protein fraction, when redissolved, was able to extract the IgA protease precursor from the cell, indicating that the extracting agent is a protein.

To isolate the extracting agent from whey, classical protein purification methods were used. First, the whey was fractionated on a DE-52 anion exchange column, with the desired activity appearing in the non-binding fraction. Proteins from the DE-52 column flowthrough then were separated by size on a BioGel P-200 gel filtration column. From the final pooled column fractions containing the desired activity, western blot analysis was used to identify lactoferrin as the agent which causes extraction of the IgA protease precursor.

Confirmation that Lactoferrin is the Extracting Agent: To confirm that lactoferrin is the extracting agent, lactoferrin from several sources was assayed for its ability to extract the IgA protease precursor. Commercially available substantially purified, but active, human and bovine lactoferrin each were able to extract the IgA protease precursor from the outer

membrane of HI. The ability of human lactoferrin (Sigma Cat. No. L-0520) to extract the IgA protease precursor is illustrated in Fig. 2. In this example, *iga* mutant HI cells were grown to mid-log phase in BHI medium, then incubated in human lactoferrin at a concentration of 4 mg/ml or 2 mg/ml. Like whey, substantially purified human lactoferrin was able to extract the protease precursor from the cell membrane. In contrast, incubation of the cells in TBS buffer did not result in release of the protease precursor from the cell membrane.

Recombinant lactoferrin was used to confirm that lactoferrin extracts the IgA protease precursor from cells. This recombinant lactoferrin was produced in eukaryotic cells, and is iron-saturated. In this experiment, HI *iga* mutant cells were grown to mid-log phase in BHI, then incubated for 1 hour in 0.5 mg/ml recombinant lactoferrin. As illustrated in Fig. 3, recombinant lactoferrin also extracted the entire IgA protease precursor from the outer membranes of wild type (HI Rd^-) and two mutant strains (HI 3-13 and HI 2-25DK) of HI. Interestingly, the IgA protease precursor extracted with lactoferrin from each of the above sources, including recombinant lactoferrin, also exhibited a decreased mobility by SDS-PAGE, providing further evidence that a milk-derived adduct is not responsible for the apparent molecular weight.

Human plasma containing serum transferrin and purified serum transferrin were also tested for their ability to extract the IgA protease precursor from cell membranes. Serum transferrin is approximately 20% as effective as lactoferrin in extracting the IgA protease precursor from the membrane, suggesting that the iron-transporting property of these proteins is not the

dominant factor responsible for extraction of the protease precursor.

The ability of lactoferrin to extract the IgA protease precursor from a cell membrane is sensitive to heat, pH, and the presence of chelators such as EGTA. Extraction of the IgA protease precursor was not detected when the whey was treated at 56°C for 30 minutes or 90°C for 5 minutes prior to incubating the whey with the HI cells. These data indicate that lactoferrin activity is lost by heating the protein (e.g., by pasteurizing milk). Similarly, the lactoferrin activity is inhibited by treating the lactoferrin with EGTA. In addition, adjusting the pH of the lactoferrin to a pH of 4.0 or below for 10 minutes, followed by neutralization of the pH, abolishes the ability of the lactoferrin to extract the IgA protease precursor. Accordingly, we have found that bovine milk that has been pasteurized or acidified (e.g., in removing casein) lacks this desired activity of lactoferrin. In addition, iron resaturation of acidified lactoferrin does not restore this activity of lactoferrin. However, fresh bovine milk is able to extract the IgA protease precursor from the outer membrane.

Processing of the Extracted Protease Precursor: Processing of the extracted protease precursor into the mature protease is inhibited by secretory IgA1 (sIgA1). This result is illustrated in Figs. 4A-C. In this experiment, HI cells were grown to mid-log phase in BHI, and 0.6 ml aliquots were incubated at 37°C for 45 minutes in milk, sIgA1-depleted milk, or sIgA1-depleted milk supplemented with sIgA1. Following the incubation in milk, the cultures were centrifuged, and the cell-free milk supernatants were further incubated at 37°C for various lengths of time (0.75 to 24 hours) (Figs. 4A-C). The IgA protease precursor subsequently was detected by

western blot analysis using antibodies directed against the IgA protease. These results indicate that the IgA protease precursor remains substantially unprocessed in milk containing sIgA1.

In contrast, the protease precursor is processed over time when it is extracted into milk that lacks sIgA1 (i.e., jacalin-treated milk) (Fig. 4B). The addition of sIgA1 to sIgA1 depleted milk blocks processing of the protease precursor. Additional experiments indicate that bovine IgG obtained from milk of a cow immunized with the IgA protease can replace the sIgA of human milk and block processing of the protease precursor. In sum, these data indicate that lactoferrin can be used in conjunction with antibodies to extract the protease precursor from the cell membrane and inhibit its processing in the mature enzyme.

Additional data indicate that the combination of lactoferrin and antibodies directed against the IgA protease is sufficient to inhibit the ability of the extracted IgA protease to cleave IgA1. Here, HI cells were grown to mid-log phase, washed with saline, then aliquotted into 0.3 ml cultures. The cultures were incubated for 1 hour in 0.2 ml of milk, IgA depleted milk, or IgA depleted milk supplemented with IgA. The cells were pelleted and washed with saline. The IgA protease activity in 30 µl aliquots of the supernatants was detected by assaying the supernatants for cleavage of radioactively labeled IgA1. As is illustrated in Fig. 4C, the combination of lactoferrin and IgA antibodies blocks the IgA protease from cleaving IgA1.

Use of Lactoferrin to Extract IgA Protease From a Gram Positive Bacterium: To determine whether lactoferrin also is able to extract the membrane-bound IgA protease from gram positive bacteria, we measured the ability of lactoferrin to extract the IgA protease of

Streptococcus sanguis. The *S. sanguis* IgA protease lacks substantial homology to HI IgA protease, and differs from that of HI in that it is a metalloprotease rather than a serine protease. After 6-8 hours of growth, 50% of the IgA protease of this bacterium stays on the cell, and 50% is released into the culture supernatant. The membrane-bound protease is thought to play a role in initiation of attachment and colonization of other pathogenic bacteria on the surface of saliva-coated teeth. We have discovered that the addition of lactoferrin to a culture of *S. sanguis* causes approximately 70% of the IgA protease to be released in 1 hour; by contrast, only 10% of the protease is released when cells are grown for 1 hour in the absence of lactoferrin. These data thus indicate that the lactoferrin effect is not limited to gram negative bacteria, as lactoferrin is able to remove the protease from *S. sanguis*. Once extracted, the activity of the protease can be inhibited with antibodies (e.g., antibodies raised against the extracted and gel-purified protease) according to the invention.

IgA Precursor-like Proteins: Proteins from several other bacteria also are functionally or structurally related to the HI IgA protease precursor, and lactoferrin is expected to extract these IgA protease precursor-like proteins from cell membranes. For example, the precursor-like protein of vacuolating cytotoxin of *Helicobacter pylori* has a helper section, and the protein is substantially similar to IgA protease. Extraction and neutralization of this toxin can protect against conditions, such as chronic gastritis, which are attributed to *H. pylori*.

Similarly, the Tsh protein of chicken *E. coli* shares over 60% sequence identity with HI IgA protease precursor. Like the IgA protease precursors, the Tsh protein includes a helper section. Tsh serves as an

agglutinin, facilitating attachment of chicken *E. coli* to epithelial cells. Thus, extraction of this protein with lactoferrin alone, or in combination with antibodies directed against Tsh (e.g., antibodies raised to the extracted and gel-purified protein), is a means for inhibiting septicemia caused by this pathogen in animals.

Also included is the HAP protein of HI. This protein is associated with the ability of the organism to bind and enter host epithelial cells. The protein shares sequence identity with the catalytic domain of IgA protease and with Tsh protein. The HAP outer membrane protein is processed in a manner similar to that of IgA protease. Extraction of any of these proteins, combined with binding of the protein with antibodies (e.g., antibodies raised against the extracted and gel-purified protein), is expected to inhibit the pathogenic effects of these bacteria (e.g., by precipitating the HAP protein).

Specificity of the Lactoferrin Effect: We next examined whether lactoferrin specifically extracts IgA protease precursor-like proteins from the outer membranes of cells, or if other cell surface proteins also are extracted. To this end, proteins on HI cells were labeled with ^{35}S-methionine, and the cells were treated with lactoferrin. The IgA protease precursor constituted approximately 80% of the labeled protein that was extracted from HI. When a similar labeling experiment was performed with an *iga*$^-$ mutant of HI which does not express the IgA protease, virtually no extracted proteins were detected. These data suggest that, in HI, the effects of lactoferrin are specific for IgA protease precursor-like proteins.

ANTIBODIES

Combined Effect of Lactoferrin and Antibodies which Bind IgA Protease- or Protease Precursor-Like Proteins: The invention provides infant formulas which inhibit bacterial IgA proteases from cleaving human IgA1, the only known substrate for this class of proteases. Formulations that include lactoferrin and antibodies that inhibit IgA proteases or IgA protease precursors or related proteins also can be used to inhibit the pathogenic effects of bacteria expressing these proteins in adults.

As is reported above, lactoferrin extracts IgA proteases or protease precursor-like proteins from the outer membranes of pathogenic bacteria. Once extracted, the proteases or protease precursor-like proteins are targeted by antibodies provided in the formula or formulation. Useful antibodies are those which specifically bind the protease precursor or protease precursor-like protein or a processed protease or protease-like protein. Antibodies that bind the precursor-like protein prevent processing of the precursor-like protein, while antibodies that bind the protease-like protein directly inhibit the activity of the protease. Accordingly, the antibodies which are used in the invention can be raised against all or a portion of the mature IgA protease or protease-like protein, or all or a portion of the IgA protease precursor or protease precursor-like protein. Many, if not all, antibodies raised against such proteins will be able to inhibit the ability of the protease precursor to process itself, or the ability of the protease to cleave its target molecule (e.g., IgA1). Methods for preparing appropriate antibodies are described below.

Method of Producing Antibodies That Specifically Bind IgA Protease- or Protease Precursor-like Proteins: To produce the infant formulas, infant formula additives, or pharmaceutical formulations of the invention, a non-human mammal, e.g., a cow, is immunized with a bacterial IgA protease precursor or protease precursor-like protein or a peptide fragment thereof (e.g., the mature IgA protease). In general, bovine immunization to obtain milk, or preferably colostrum, containing antibodies is preferably performed as follows. During the last month of gestation, the cow transfers a very large amount of circulating plasma IgG into its udder. A parenteral (subcutaneous, intramuscular, etc.) injection of an IgA protease (or the preferred antigen) is preferably made during this period to stimulate high levels of circulating (plasma) antibody that eventually are transferred to the colostrum and milk.

This colostrum is then processed by conventional methods to produce the infant formula, formula additive, or pharmaceutical formulation. Because the desired activity of lactoferrin is sensitive to heat and acidification, processing steps which involve heat or acidification should not be carried out after lactoferrin is added. When a person (e.g., an infant) drinks the formula containing the antibody and lactoferrin, the formula protects the mucosa in all of the areas where ingested milk normally contacts the mucosa, i.e., the oral cavity, nasopharynx, bronchial passages, and intestine. Because simple ingestion of maternal milk normally is sufficient to deliver antibodies to these mucosal surfaces, ingestion of the formula of the invention is expected to provide an adequate means for delivery of the formula to the preferred mucosa.

In one example, pregnant cows received two injections of IgA proteases mixed with 1 mg of the

adjuvant Quil A. Each immunization employed 3 ml saline and 1 mg IgA protease (or 6 mg IgA protease for a "cocktail"). Injections were given approximately 28 and 14 days before expected calving. Inoculation was in four sites: 1 ml injected subcutaneously into each shoulder, and 0.5 ml injected intramuscularly into the gluteal region on each side). The maternal colostrum (approximately four liters) then was collected by venipuncture (bovine colostral whey proteins typically consist of 40% immunoglobulins, 35% beta-lactoglobulin, 15% lactalbumin and small amounts of albumin and other proteins). The colostrum was centrifuged for 30 minutes at 12,000 x *g* at 4°C to remove the fat (which floats to the top of the sample). The casein then was precipitated by incubating the colostrum for 1 hour with 15 units of rennin. The curd subsequently was tightened by heating the sample to 56°C for 10 minutes, and the casein was removed by centrifugation at 4°C for 25 minutes at 12,000 x *g*. If desired, the colostrum may be frozen at this point.

To produce the formulas and formulations of the invention, active lactoferrin is added to the processed colostrum or milk to a concentration of 0.01-10 mg/ml, preferably 0.5-1.0 mg/ml. After lactoferrin is added, the pH of the formula should not be permitted to drop below pH 4.0, preferably pH 5.0, and the formula should not be heated significantly (e.g., 90°C for 5 minutes, or 56°C for 30 minutes). Generally, lactoferrin is active at a temperature 18-37°C.

The colostrum or milk may be tested by culture for sterility, and it can be frozen until used.

Monoclonal IgA Protease Antibodies: Monoclonal antibodies directed against bacterial IgA protease precursor-like proteins also can be used according to the invention. These antibodies or antibody fragments can be

added to infant formulas in the same way as the bovine antibodies or bovine antibody containing colostrum described above.

Monoclonal antibodies useful in the invention can be made by standard techniques by immunizing mice with all or a portion of an IgA protease precursor-like protein, fusing the murine splenocytes with appropriate myeloma cells, and screening the antibodies produced by the resultant hybridoma lines for the requisite ability to bind the IgA protease precursor-like protein (e.g., by means of an ELISA assay). A subsequent screening may be necessary to select binding antibodies that also inhibit or remove the protease or protease precursor. Monoclonal antibody production and screening can be performed according to Uchiyama et al. (*J. Immunol.* 126:1393, 1981). Alternatively, useful antibodies may be isolated from a combinatorial library produced by the method of Huse et al. (*Science* 246:1275, 1989).

The invention can employ not only intact polyclonal or monoclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or $(Fab)_2$ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Patent No. 4,946,778); or a chimeric antibody, for example, an antibody that contains the binding specificity of a murine or bovine antibody, but in which the remaining portions are of human origin.

IgA Protease Inhibition Assay: A suitable enzyme assay uses human myeloma IgA1 immunoglobulin trace labelled with ^{125}I as a substrate. Fragmentation products of the IgA1 heavy chain are measured by first separating these fragments on polyacrylamide gels. A suitable antibody inhibits protease activity by binding to the protease precursor or the protease.

In this example, the enzyme activity is assayed. Generally, the assay involves quantitating the amount of IgA1 Fab produced over time. The Fab produced can be measured by measuring release of the Fd fragment, which is the heavy chain of the Fab region. A typical reaction mixture has a volume of 75 μl, consisting of 25 μl stock substrate solution (2 mg/ml purified IgA1 with 2% labeled IgA1), 25 μl of a colostrum dilution containing the antibody (or a Tris/HCl/BSA buffer control), and 25 μl active IgA protease (*Haemophilus, Neisseria* or *Streptococcal* species, but any IgA protease can be used). After the enzyme and antibody were incubated for 30 minutes at 37°C to allow them to interact, the IgA1 substrate was added, and incubation continued at the same temperature. Aliquots of 10 μl were removed at 10-minute intervals over a 40 minute period and hydrolysis of IgA1 measured as follows.

The aliquot was added to 100 μl of sample buffer for SDS-PAGE, and boiled 5 minutes. The sample buffer contains 12.5% glycerol, 1.2% sodium dodecyl sulfate, 1.2% mercaptoethanol, and 0.001% bromphenol blue dye. Aliquots of 100 μl were electrophoresed on 9% PAGE gels after which the gels were stained with 0.05% Coomassie Brilliant Blue, dried, and autoradiographed at -70°C using Kodak XAR5 film with an intensifying screen. The developed autoradiograph was used as a template for cutting the stained and dried gel into segments consisting of 1) any uncleaved IgA heavy chain and 2) the 22 kD Fd alpha fragment representing that portion of the heavy chain within the Fab fragment digestion product. Radioactivity in the uncleaved heavy chain and in its Fd product were counted on a Beckman Biogamma Counter, background counts from control digests were subtracted from each value, and quantitation of IgA protease

activity was expressed as a percentage of the heavy chain that has been cleaved using the formula:

$$\frac{\text{cpm Fd}}{\text{cpm Fd + cpm heavy chain}} \times 100$$

An inhibition assay from the colostrum of 18 cows is shown in Fig. 5. The titers for 18 cows immunized with individual IgA proteases for the first time during a single calving are provided. As is suggested by the figure, the preferred antigens are IgA proteases of *Neisseria* and *Haemophilus*. Inhibiting antibodies were detected in milk obtained after a second immunization and second calving (Fig. 6). The levels of inhibition obtained with colostrum are higher than those obtained with milk, and antibodies from the second calving are more potent than those obtained with the first calving

To determine whether the difference observed between milk and colostrum is due to a difference in the IgG concentrations in the samples, the samples were normalized for IgG level. Even when IgG levels are accounted for, colostral antibodies were approximately 6 to 10-fold more inhibitory than were milk antibodies.

Fig. 7 provides a second set of examples of IgA1 protease inhibition activities from serum, colostrum, and milk after a first lactation. In this example, inhibiting antibodies were produced when the immunizing antigen was HIe, HIb, *N. meningitidis* 1C or 2R. Generally, antibody titers were greatest in colostrum. Inhibiting antibodies were also obtained in serum at 14 or 21 days after calving (Fig. 7).

Experiments also show that the bovine colostral antibodies of the invention differ in inhibiting titre against various IgA proteases. Fig. 8A shows that enzymes from strains sharing the same serogroup with that of the source of the immunizing enzyme (i.e., "serotype

correct" assays) are better inhibited than are enzymes from non-matched serogroups. For example, cow #1, immunized with type 1 protease of HI strain Rd, inhibited three out of three serotype d strains (HiRd, HiD2, and HiD3) with a reciprocal titer of approximately 2,000 (at an IgA protease activity of 50%). IgA proteases from HI of other serotypes (groups b (HiB), e (HiE), f (HiF), and C (HiC)) were inhibited less than 100 at the same level of activity. Fig. 8A shows that enzymes having the same cleavage type as that of the immunogen are better inhibited. Thus, it is preferable to use several enzymes to immunize each cow such that various, e.g., *Haemophilus*, proteases are blocked. Alternatively, antibodies from cows immunized with each protease can be combined for addition to infant formula.

Fig. 8B shows the inhibitory curves of cow #236, which was immunized with type 2 IgA protease of HI serotype e. The reciprocal inhibitory titer was 3180. In this case, the three proteases having type 2 specificity (HiC 3 (2), HiE 27 (2), and HiE (2)) were inhibited to a greater extent than the three proteases having type 1 specificity. These curves in Fig. 8B show that in addition to serogroup specificity as shown in Fig. 8A, certain of the bovine antibodies of the invention also show more specificity for enzymes of the correct cleavage type.

Protease-inhibition titers of cows after a second set of immunizations are provided in Fig. 9. Cow #265 had a maximum serum titer at day 21 (titer = 80), but it showed a 4-fold increase in colostrum titer relative to the first lactation (titer = 640). Cow #337 immunized with *N. meningitidis* 2R IgA1 protease had a serum inhibitory titer at day 0 (titer = 40) which increased by day 14 (titer = 80), and reached a maximum at day 21 (titer = 320). After a second calving, this cow had a

colostrum titer of 1280 and a milk titer of 20, which were the highest levels of all the cows, even though no inhibition was detectable after the first calving.

Characterization of Bovine IgA Protease Antibody: Using an anti-bovine IgG antiserum we have shown that the IgA protease antibody in bovine colostrum is IgG. This was done by exposing enzymes on western blots to the colostral preparations as a first antibody, and examining the isotype of the binding antibody by various antibodies against cow serum. Furthermore, chromatography of the inhibiting colostrum on Biogel P200 columns shows that the ability to inhibit co-elutes precisely with the IgG peak.

The inhibiting property of bovine colostral IgG is localized in the Fab fragment (as anticipated for an antibody). This was shown by using papain to cleave the inhibiting protein into its various fragments as follows. Papain (enzyme/protein ratio of 1/100 w/w) was added to bovine colostral immunoglobulins and the mixture incubated at 37°C for 16 hours. Proteolysis was stopped with Ca^{++} added in excess, and the material examined on western blot analysis; this showed about 80% hydrolysis. The Fab fragments were isolated by column chromatography on Biogel P100, and identified by their reduced molecular weight (changed elution position) and light chain content as determined by appropriate antisera.

This Fab fragment material was tested as an IgA protease inhibitor, and found to be 2-3 fold less effective than the starting material before proteolysis. Thus, the entire IgG antibody molecule is not required for IgA protease inhibition, although some inhibiting power could be lost by isolation of the monovalent Fab region of the antibody.

FORMULAS AND FORMULATIONS

Lactoferrin and non-human antibodies to bacterial IgA protease-like proteins or protease precursor-like proteins, e.g., bovine IgG capable of inhibiting bacterial IgA proteases, can be added to bovine milk-based infant formulas. Like human milk, such formulas will inhibit IgA protease-like proteins and protease precursor-like proteins in the fluids bathing the nasopharynx and upper respiratory passages of the infant. The advantage of such inhibition is that, in general, the child's own IgA1 will be protected from cleavage by the IgA proteases. Thus, all IgA1 antibodies will benefit, not only those directed against the IgA1 protease producing bacteria themselves.

The processed colostrum containing the antibodies can be diluted or concentrated and used in combination with lactoferrin as an infant formula additive. Standard infant formulas include the SIMILAC® line of formulas (Ross Laboratories, Columbus, Ohio) and the SMA® line of formulas (Wyeth Laboratories, Philadelphia, Pennsylvania). Furthermore, the colostral antibodies plus lactoferrin also can be used undiluted as an infant formula, with or without added nutrients. The bovine antibodies to bacterial IgA proteases can also be purified from the colostrum and added along with lactoferrin to infant formula directly. For example, the antibodies and lactoferrin may be dried into a powder form to be added to formulas, or even maternal milk.

To determine whether the inhibitory effect of the bovine antibodies against IgA protease precursor was maintained when added to infant formulas, *Haemophilus influenzae* type 1 protease antibodies were added to various infant formulas including SIMILAC®, Enfamil®, S.M.A.®, ProSobee®, and NUTRAMIGEN® (Mead-Johnson, Evansville, Indiana). Titers were done as for the

colostral titers described above. The results showed that the formulas supplemented with these antibodies had essentially the same inhibitory effect as the colostral whey preparation described above.

Figs. 10A and 10B are autoradiographs of polyacrylamide electrophoresis gels of ^{125}I-IgA1 substrate subjected to IgA protease. These autoradiographs show uncleaved (IgA heavy chain) substrate, and fragments arising from proteolysis (Fab). In all cases the protease assay was carried out for 30 minutes.

Fig. 10A shows that IgA protease is active when added to five commercial infant formulas, whereas Fig. 10B shows that the IgA protease is inactive when added to formula supplemented with the inhibiting bovine antibodies of the invention. The colostrum of cows never immunized with proteases, which lacks antibody, was used as a control.

Fig. 10A shows the gel resulting from *Haemophilus influenzae* type 1 IgA protease added to a number of commercial infant formulas, along with trace amounts of ^{125}I-IgA substrate. The top two lanes are controls: the top lane is substrate without protease, which resulted in no cleavage products; the second lane is substrate and protease in buffer (not formula) to show the basic activity of the protease added to the formula products. The subsequent five lanes show that unmodified formulas cannot affect activity of IgA protease; the Fab fragment is the cleavage product.

Fig. 10B shows that the *Haemophilus influenzae* IgA protease used above in the gel of Fig. 10A is inactive in infant formula (here NUTRAMIGEN®) supplemented by an anti-IgA protease from immune cow colostrum. The bottom two lanes are substrate and enzyme controls. The top four lanes are NUTRAMIGEN® formula containing varying amounts of cow #1 immune colostrum: from top to bottom,

the ratio of colostrum:formula (vol:vol) is 1:640, 1:1280, 1:2560, and 1:5120. The enzyme added to all these dilutions is inactive, as shown by the inability to fragment any ^{125}I-IgA substrate during 30 minutes.

In general, the bovine antibodies of the invention should be fed to an infant in combination with lactoferrin, preferably as a formula supplement, at a dosage of about 1.0 to 2.0 grams of bovine colostral antibody protein/kg/day and 0.01 mg/ml to 10 mg/ml of lactoferrin/kg/day, preferably 0.5-1.0 mg/ml, equally distributed over all feedings. This dosage must be adjusted dependent on the titer of the antibodies in the particular colostral preparation used as the infant formula additive, because different colostrum preparations have different percentages of the IgA protease antibodies compared to total antibodies in the preparation.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

For example, the invention includes formulations that are made without milk, but which include lactoferrin and antibodies that inhibit the proteolytic activities of IgA proteases, protease precursors, protease-like proteins, and protease precursor-like proteins. Non-milk based formulations that include antibodies and lactoferrin can be used as infant formulas. Milk or non-milk formulations of the invention can be used to inhibit infections in adults. For example, *S. sanguis* is a major

initiator of plaque formation which causes dental caries in adults. Lactoferrin and inhibiting antibodies can thus be formulated into a mouthwash or beverage, for example, for use in adults. The formulations can also be used in treating *Pneumococcus* infections in adults and children. These bacteria produce IgA proteases and are major lung pathogens, causing pneumonia in adults. Accordingly, the invention can be formulated for intrabronchial or intranasal administration. In addition, *Haemophilus influenza* also infects adults, and the invention thus can be used to inhibit HI IgA proteases in adults as well.

What is claimed is:

1. An infant formula comprising
pasteurized milk,
active lactoferrin, and
an antibody that specifically binds either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor.

2. A formula of claim 1, wherein said antibody specifically binds an IgA protease.

3. An infant formula of claim 1, wherein said milk is non-human colostrum.

4. A formula of claim 3, wherein said colostrum is bovine colostrum.

5. A formula of claim 1, wherein said antibody is bovine IgG.

6. A formula of claim 1, wherein said lactoferrin is human lactoferrin.

7. A formula of claim 1, wherein said lactoferrin is non-pasteurized lactoferrin.

8. A method for preparing a pasteurized infant formula, said method comprising
immunizing a non-human mammal with all or a portion of an IgA protease or an IgA protease precursor;
collecting from said mammal milk comprising antibodies that specifically bind either an IgA protease or an IgA protease precursor, or both an IgA protease and an IgA protease precursor;
pasteurizing said milk; and

adding active lactoferrin to said milk to prepare said infant formula.

9. A method of claim 8, wherein said lactoferrin is human lactoferrin.

10. A method of claim 8, wherein said lactoferrin is non-pasteurized lactoferrin.

11. A method for producing an infant formula additive, said method comprising

immunizing a pregnant non-human mammal with all or a portion of an IgA protease or an IgA protease precursor;

collecting from said mammal milk comprising antibodies which specifically bind either an IgA protease, an IgA protease precursor, or both an IgA protease and an IgA protease precursor;

pasteurizing said milk; and

mixing pasteurized milk with active lactoferrin to produce the infant formula additive.

12. A method of claim 11, wherein, prior to said mixing, fat and casein are removed from said milk.

13. A method of claim 11, wherein said lactoferrin is non-pasteurized lactoferrin.

14. A method for inhibiting proteolysis of an IgA1 antibody by an IgA protease, said method comprising

contacting a cell comprising a precursor of said IgA protease with a formulation comprising

a pasteurized excipient, and

active lactoferrin, thereby producing an extracted IgA protease precursor; and

contacting said extracted IgA protease precursor with an antibody which specifically binds said IgA protease.

15. A method of claim 14, wherein said lactoferrin is non-pasteurized lactoferrin.

16. An antibody that specifically binds an IgA protease precursor and that does not specifically bind an IgA protease.

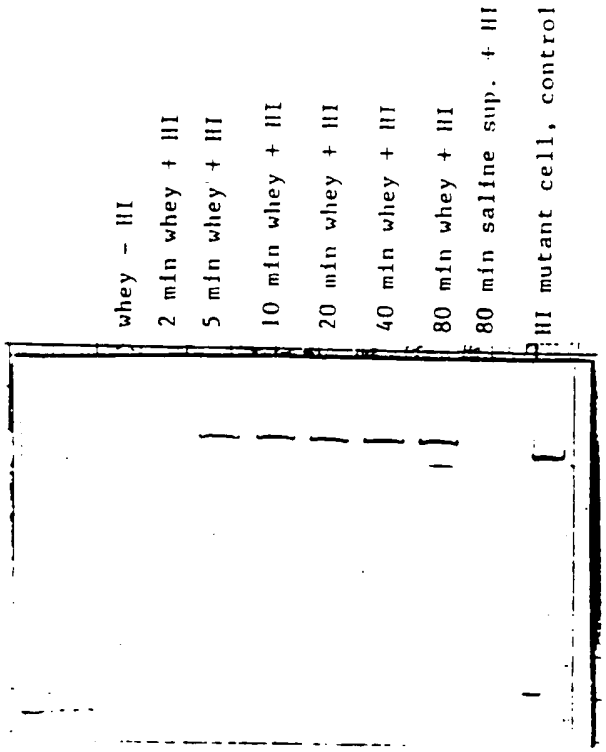
17. The antibody of claim 16, wherein said antibody specifically binds the helper section of said IgA protease precursor.

18. A pharmaceutical formulation comprising active lactoferrin and an antibody which specifically binds either an IgA protease-like protein, or an IgA protease precursor-like protein, or both an IgA protease-like protein and an IgA protease precursor-like protein.

19. A formulation of claim 18, wherein said lactoferrin is non-pasteurized lactoferrin.

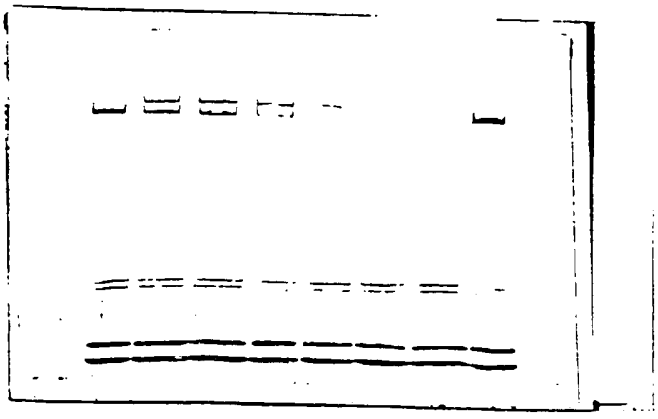
20. The formulation of claim 18, wherein said antibody specifically binds an IgA protease-like protein selected from the group consisting of vacuolating cytotoxin, Tsh protein, and HAP protein.

Fig. 1A



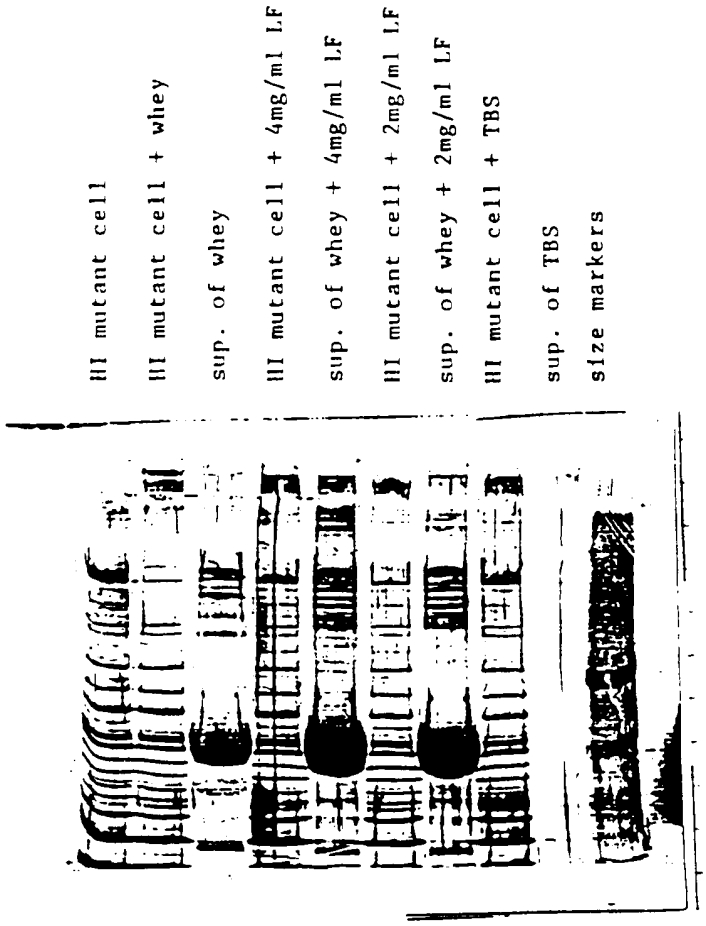
whey supernatant

Fig. 1B



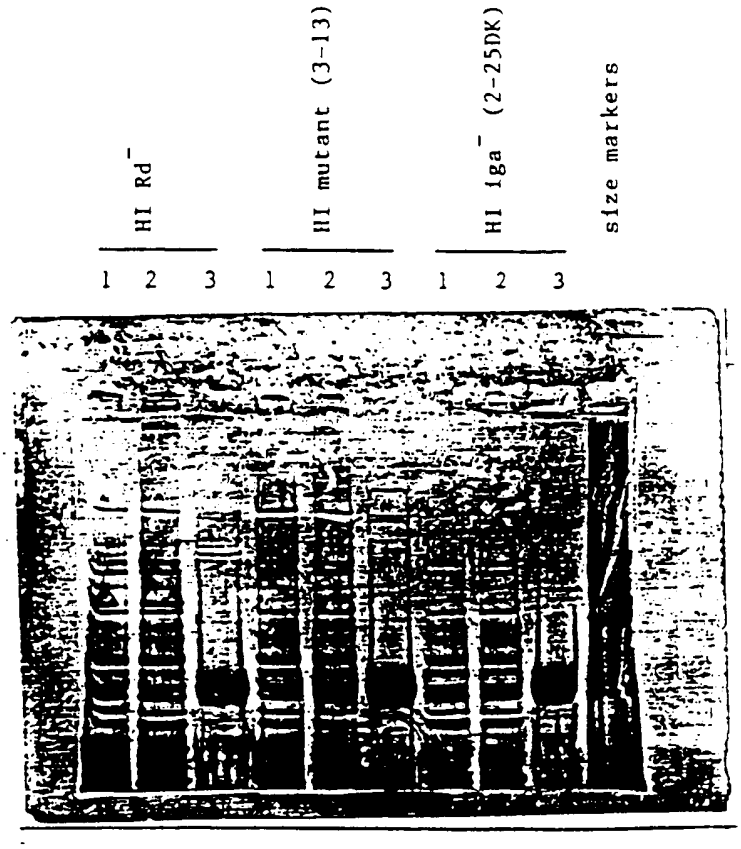
HI mutant cell pellet

FIG. 2



LF: lactoferrin

FIG. 3



1. cells after 5 hrs. growth in BHI
2. cells after 5 hrs. growth in BHI, followed by 1 hr. growth in recombinant lactoferrin
3. recombinant lactoferrin supernatant after 1 hr. cell growth

Fig. 4A

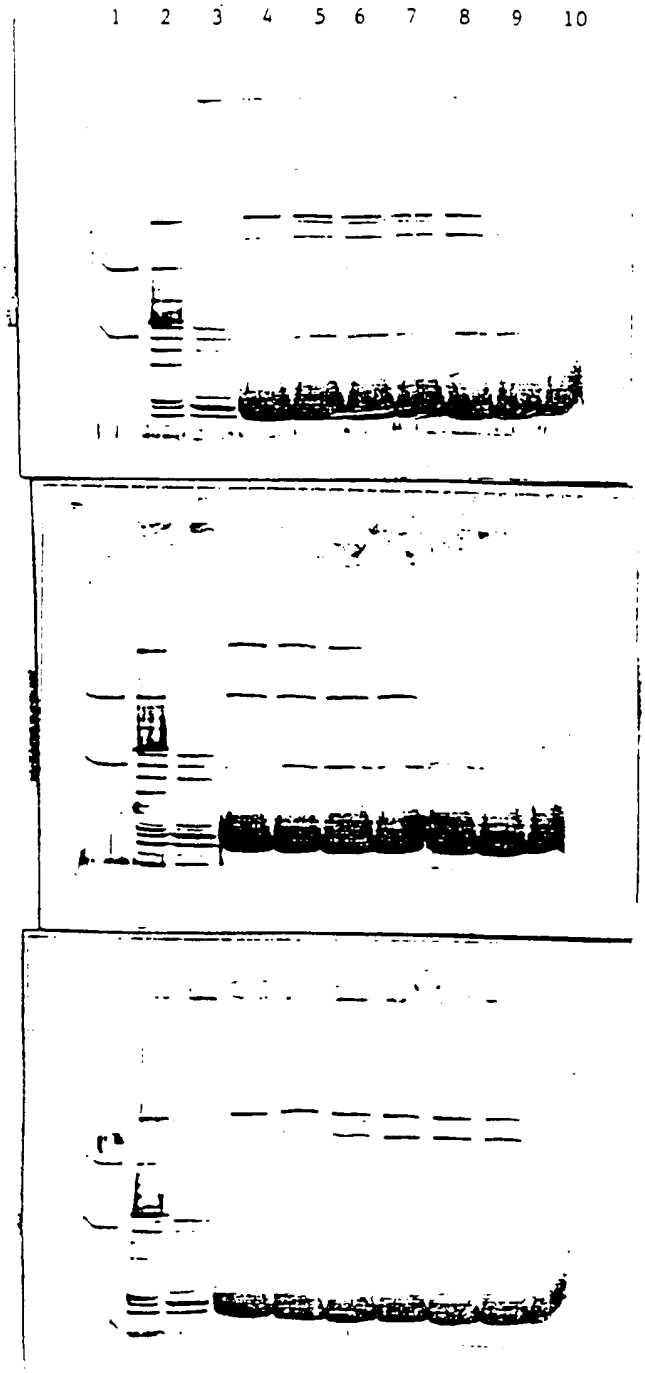
Milk

Fig. 4B

sIgA1 depleted milk

Fig. 4C

sIgA1 depleted milk supplemented with sIgA1



1. Culture supernatant of HI Rd^- in BHI
2. Rd^- cell in BHI
3. Rd^- cell after growth in milk, sIgA1 depleted milk, or sIgA1 depleted milk supplemented with sIgA1
4. Milk sup. 0 min
5. Milk sup. 45 min
6. Milk sup. 90 min
7. Milk sup. 180 min
8. Milk sup. 9 hrs.
9. Milk sup. 24 hrs.
10. Milk only.

FIG. 5

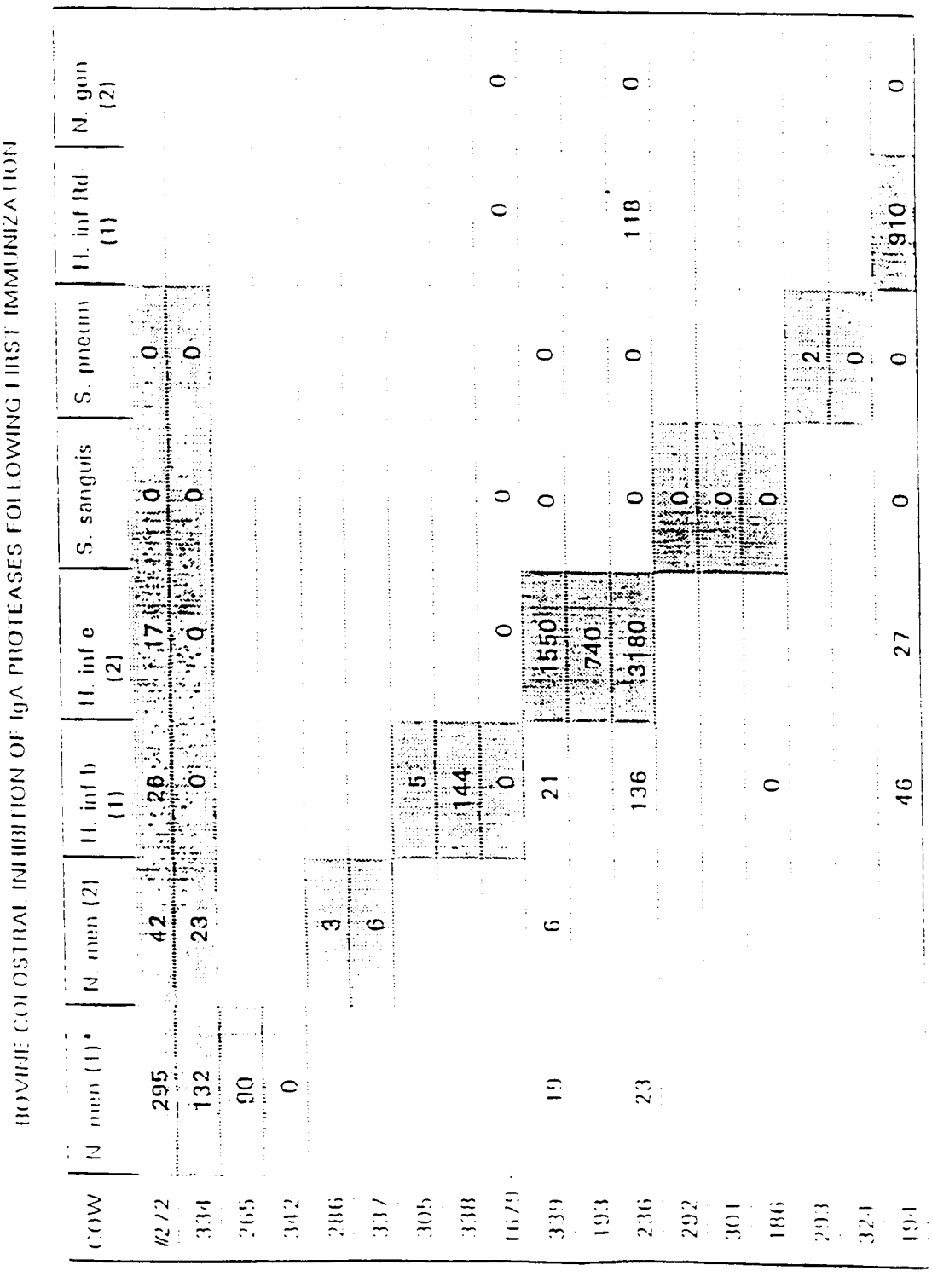

BOVINE COLOSTRAL INHIBITION OF IgA PROTEASES FOLLOWING FIRST IMMUNIZATION
COW
N. men (1)*
N. men (2)
H. inf b (1)
H. inf e (2)
S. sanguis
S. pneum
H. inf Rd (1)
N. gon (2)
#272
295
42
26
17
0
0
0
0
334
132
23
0
0
0
0
0
0
265
90
0
342
0
3
286
6
337
5
305
144
338
0
0
0
0
0
0
1679
19
6
21
1550
0
0
0
0
339
740
193
23
136
3180
0
0
118
0
236
292
301
0
186
293
2
321
46
27
0
0
910
194
*Number in parenthesis refers to enzyme cleavage type

FIG. 6

BOVINE ANTIBODY AGAINST IMMUNIZING PROTEASE

FOLLOWING SEQUENTIAL CALVINGS

(Reciprocal of IC_{50} titre)

Cow	Colost. calf #1	Colost. calf #2	Milk* (1 Wk)
265	90	13,500	145
342	0	8,800	100
337	6	54,000	360
339	1,550	12,400	36
293	2	55	0
324	0	0	0
292	0	6	0
301	0	0	0

Antigens used...
265 *N. meningitidis* type 1
342 *N. meningitidis* type 1
337 *N. meningitidis* type 2
339 *H. influenzae* type 2
293 *S. pneumoniae*
324 *S. pneumoniae*
292 *S. sanguis*
301 *S. sanguis*

* Milk data are from second calving.

FIG. 7

IgA1 protease-inhibition activity[a] (ELISA) of cow serum, colostrum and milk following immunization[b] prior to a first lactation.[c]

Cow #	Immunizing[d] Antigen	Serum Day 0[e]	Serum Day 14	Serum Day 21	Whey Colostrum	Whey Milk[f]
193	*H. influenzae e*	0	10	40	160	0
339	*H. influenzae e*	0	0	40	160	0
305	*H. influenzae b*	0	0	0	0	0
338	*H. influenzae b*	0	0	10	10	0
265	*N. meningitidis 1C*	0	40	80	40	0
342	*N. meningitidis 1C*	0	160	40	0	0
286	*N. meningitidis 2R*	0	20	40	0	0
337	*N. meningitidis 2R*	0	10	20	0	0
292	*S. sanguis*	0	0	0	0	0
301	*S. sanguis*	0	0	0	0	0
293	*S. pneumoniae*	0	0	0	0	0
324	*S. pneumoniae*	0	0	0	0	0

[a] reciprocal of the last dilution in which optical density is two standard deviations above the negative control

[b] cows were immunized on days 0, and 14 prior to expected calving date

[c] refers to the first lactation in which these cows were observed in this study, but does not necessarily reflect actual cow parity

[d] antigens were semi-purified IgA1-cleaving proteases of each bacterium listed

[e] relative to expected calving date (day 28)

[f] milk sample from one week postpartum

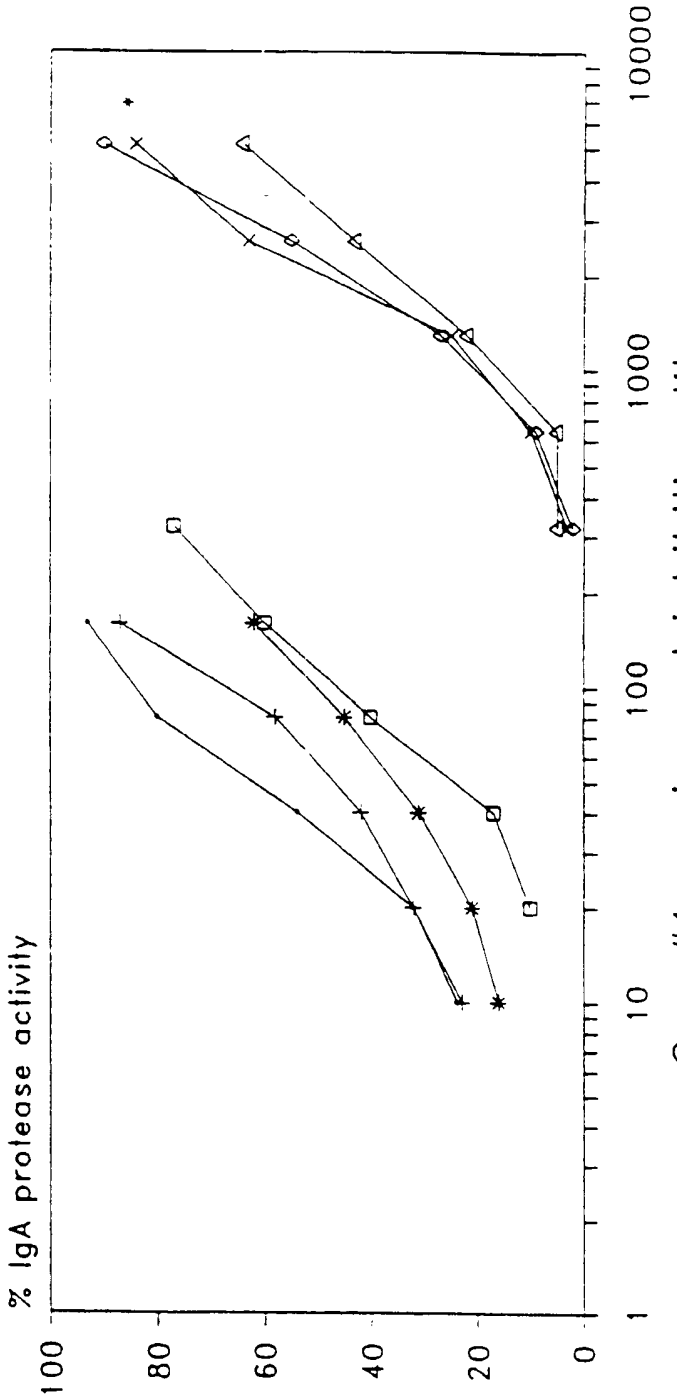

% IgA protease activity
100
80
60
40
20
0
1
10
100
1000
10000
Cow #1, reciprocal inhibition titre
HiC (2)
HiE (2)
HiB (1)
HiF (1)
HiRd- (1)
* [antigen]
HiD2(2)
HiD3 (1)

FIG. 8A

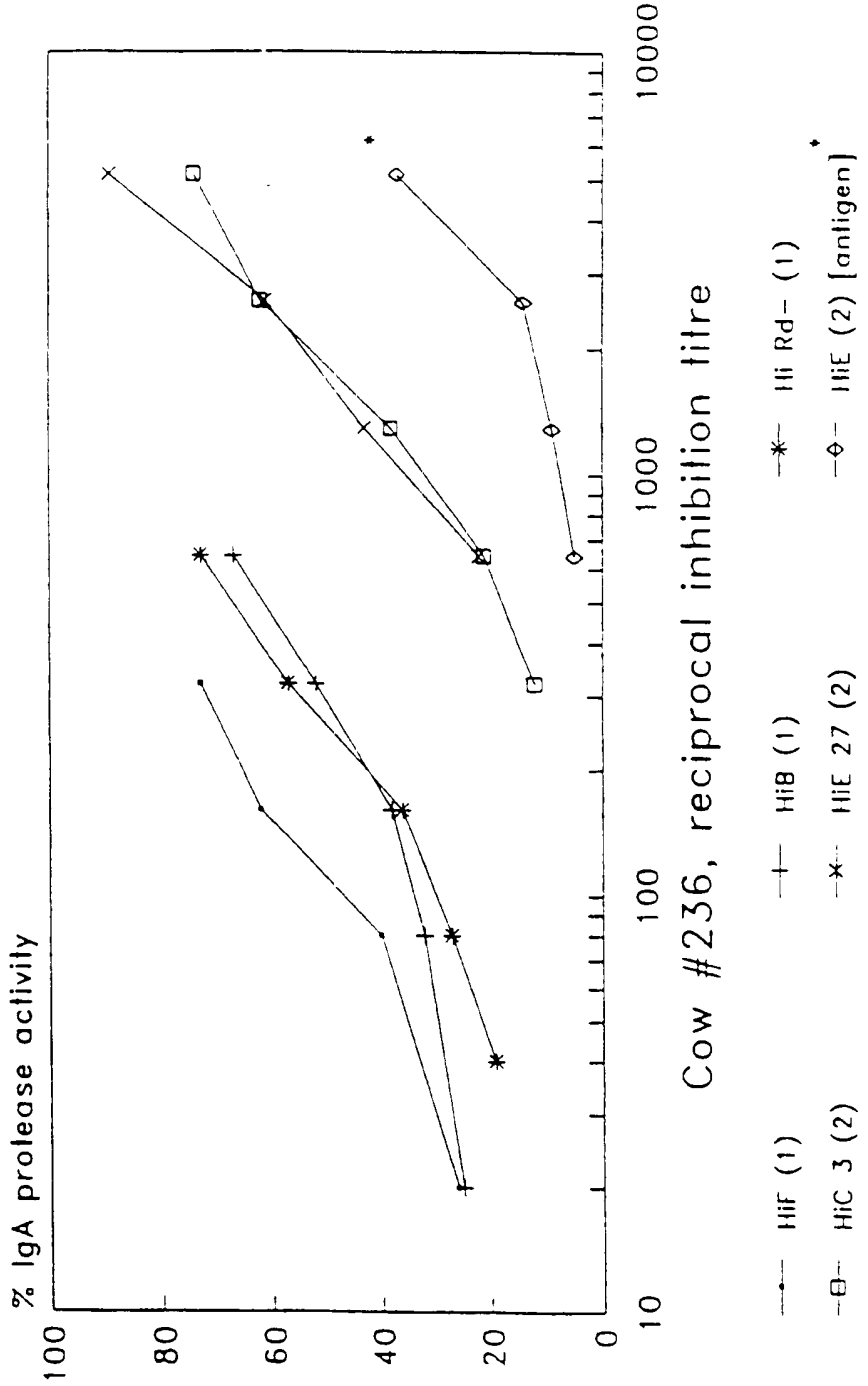

% IgA protease activity
100
80
60
40
20
0
10
100
1000
10000
Cow #236, reciprocal inhibition titre
HiF (1)
HiB (1)
Hi Rd- (1)
HiC 3 (2)
HiE 27 (2)
HiE (2) [antigen]

FIG. 8B

FIG. 9

IgA1 protease-inhibition activity[a] (ELISA) of cow serum, colostrum and milk following immunization[b] prior to a second lactation.[c]

Cow #	Immunizing[d] Antigen	Serum Day 0[e]	Serum Day 14	Serum Day 21	Whey Colostrum	Whey Milk[f]
339	*H. influenzae e*	10	20	20	320	10
265	*N. meningitidis 1C*	40	80	80	640	10
342	*N. meningitidis 1C*	40	40	320	320	10
337	*N. meningitidis 2R*	40	80	320	1280	20
292	*S. sanguis*	0	0	0	0	0
301	*S. sanguis*	0	0	0	0	0
293	*S. pneumoniae*	0	0	0	0	0
324	*S. pneumoniae*	0	0	0	0	0

[a] reciprocal of the last dilution in which optical density is two standard deviations above the negative control

[b] cows were immunized on days 0, and 14 prior to expected calving date

[c] refers to the second lactation in which these cows were observed in this study, but does not necessarily reflect actual cow parity

[d] antigens were semi-purified IgA1-cleaving proteases of each bacterium listed

[e] relative to expected calving date (day 28)

[f] milk sample from one week postpartum

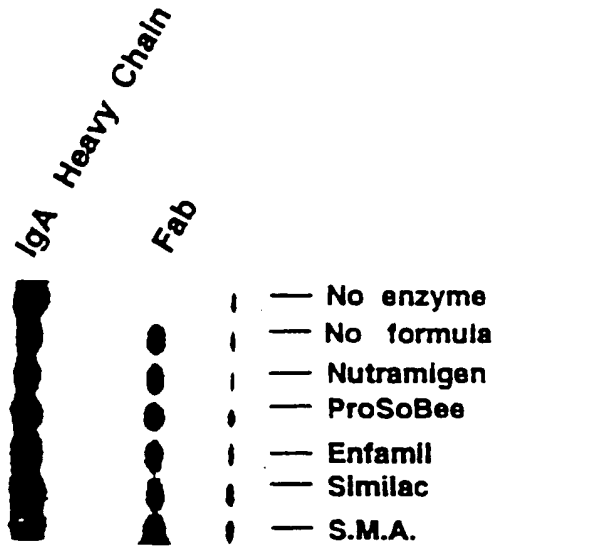


Fig. 10A

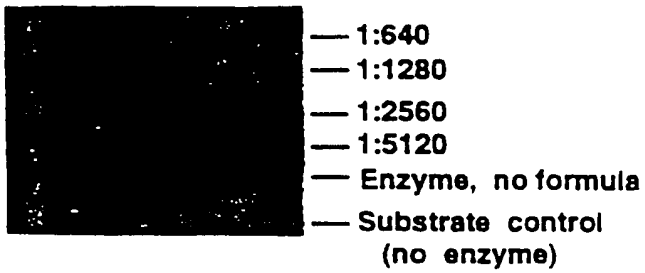


Fig. 10B

INTERNATIONAL SEARCH REPORT

Inte...ational application No.
PCT/US96/12447

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	LOMHOLT et al. Distinct antigenic and genetic properties of the immunoglobulin A1 protease produced by Haemophilus Influenzae biogroup aegyptius associated with Brazilian purpuric fever in Brazil. Infection and Immunity. 01 November 1995, Vol 63, No. 11, pages 4389-4394, see entire document.	1-2, 8, 11, 14
Y	MORELLI et al. Immunogenicity and evolutionary variability of epitopes within IgA1 protease from serogroup A Neisseria meningitidis. Molecular Microbiology. 01 January 1994, Vol. 11, No. 1, pages 175-187, see entire document.	1-2, 8, 11, 14
Y	WO 93/10818 A1 (NEW ENGLAND MEDICAL CENTER HOSPITALS, INC) 10 June 1993, see entire document.	1-5, 8, 11-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 SEPTEMBER 1996	31 OCT 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICK NOLAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12447

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WITTLER et al. Neutralization of Streptococcus-Sanguis IgA1 protease by lactoferrin and transferrin. J. of Dental Research. 10 March 1993, Vol. 72, page 327, see entire document.	1, 6-11, 13-15, 18-19

INTERNATIONAL SEARCH REPORT

Inte. .ational application No.
PCT/US96/12447

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 35/20, 38/16, 39/40, 39/095, 39/102, 39/395; C07K 1/00, 16/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 150.1, 157.1, 164.1, 165.1, 169.1, 249.1, 256.1, 535; 426/580; 514/6; 530/350, 387.1, 387.15, 3 88.4, 389.1, 389.5, 400, 832

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/130.1, 150.1, 157.1, 164.1, 165.1, 169.1, 249.1, 256.1, 535; 426/580; 514/6; 530/350, 387.1, 387.15, 3 88.4, 389.1, 389.5, 400, 832